ARTIFICIAL INSEMINATION AND SPAWNING OF PACIFIC WHITE SHRIMP *LITOPENAEUS VANNAMEI*: IMPLICATIONS FOR A SELECTIVE BREEDING PROGRAM

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ABSTRACT

Through the U.S. Marine Shrimp Farming Program, the Oceanic Institute has established a breeding program where Pacific white shrimp *Litopenaeus vannamei* are selected for rapid growth and resistance to Taura Syndrome Virus. Until recently, OI produced maternal half-sib families by mating one female with two different males within a 2-wk spawning period. The artificial insemination (AI) technique used to produce these families relied on the removal of both spermatophores from a single male and the application of the spermatophores over the thelycum of a ripe female. The female was then placed in a spawning tank where fertilized eggs were liberated. If a previously inseminated female developed ripe ovaries before the 2-wk period elapsed, she was inseminated again with spermatophores from a different male. In an effort to maximize the number of half-sib families and reduce the time that families are produced, a different AI technique was used to produce paternal half-sib families. With this technique, each of the two spermatophores from a single male was manually extruded and placed on the thelycum of two different females. This later technique resulted in a significantly higher ($P < 0.001$) spawning success (84% *vs* 58%) and females produced significantly more ($P < 0.001$) viable nauplii per spawn (24,400 nauplii *vs* 8,500 nauplii). Importantly, the time to produce selected families was reduced from 14 d to 9 d, and the number of half-sib families increased. These improvements have significant implications for a selective breeding program.

INTRODUCTION

Most shrimp cultured worldwide are either collected from the wild or offspring from wild-caught broodstock. This practice is risky because wild-caught shrimp may be carriers of pathogens, including viruses. Several of these viruses have devastated the global shrimp farming industry in recent years, resulting in the emergence of novel production systems that rely on pathogen exclusion (McIntosh 1999; Moss 1999). Additionally, there are concerns about the ecological effects of harvesting wild shrimp for aquaculture, and this activity has been implicated in changing the dominant species composition of wild shrimp caught by fishermen in coastal Ecuador (Landesman 1994).

A significant disadvantage in culturing wild-caught shrimp is the inability of the farmer to benefit from domestication and genetic improvement of stocks. Many penaeid shrimp possess characteristics that are amenable to selective breeding, including the ability to close the life cycle in captivity, a short generation time, and high fecundity. Recently, there has been an emergence of shrimp breeding programs in Asia and the Americas, and the Oceanic Institute (OI) has played a significant role in establishing some of the fundamental principles of operating such a program, including techniques of artificial insemination (AI).

Most commercial shrimp hatcheries rely on natural matings to produce larvae (described by Yano et al. 1988). Advantages of natural mates over artificial insemination are a greater number of nauplii produced per spawn and decreased labor costs. However, the male is unknown and this may be important information for selective breeding programs. The male may be identified by DNA fingerprinting if it is undesirable to use AI (Moore et al. 1999; Hetzel et al. 2000), but this approach requires sophisticated procedures and equipment that typically are unavailable to shrimp farmers. In the OI breeding program, specific males are mated with specific females by AI to produce half/full-sib families for estimation of genetic parameters, including heritability estimates, phenotypic and genetic variation, and phenotypic and genetic correlations. However, spawning success and the number of nauplii produced per

spawn are lower than with natural mates. Originally, OI operated its breeding program by producing maternal half-sib families. Production of half-sib families depended on multiple spawns of a single female within a 2-wk period. However, this approach may confound genetic analysis by introducing maternal and environmental effects. In addition, the ratio of full-sib families to number of dams was 1.2, whereas the goal of the breeding program was to obtain a ratio of 2.0. A reduction in the number of half-sib families in the breeding program results in less accurate estimates of genetic parameters. In light of the drawbacks associated with producing maternal half-sib families by AI, an experiment was conducted to compare two AI techniques in order to determine the most efficient method to produce offspring for the breeding program at OI.

MATERIALS AND METHODS

Broodstock shrimp were obtained from the shrimp production facility at OI and were negative for specifically listed pathogens (Lotz 1997). Three 4.3-m diameter maturation tanks were stocked with 70 female and 40 male Pacific white shrimp *Litopenaeus vannamei* at an initial mean weight of 54 g (SD = 9.0 g) for females and 42 g (SD = 7.0 g) for males. Prior to stocking, all broodstock were tagged with colored, numerically coded, plastic eyestalk tags (National Band and Tag Co., Newport, KY, USA) to facilitate individual identification of the shrimp. Unilateral eyestalk ablation was performed on all females within 1 wk after the first molt in the maturation system (Wyban and Sweeney 1991). Broodstock shrimp received a maturation diet consisting of enriched *Artemia*, bloodworms, and squid and were fed between 24-28% of their biomass per day. The maturation diet was provided four times daily at 0830, 1100, 1330, and 1600. Environmental conditions were standardized among the three maturation tanks; flow rates were 13-15 L/min (200% daily exchange), water temperature was 28-29 C, salinity was 33-35 ppt, and dissolved oxygen was 4-5 mg/L. Photoperiod was set for gradual sunrise at 0300 and sunset at 1600 (13 h light and 11 h dark). Sourcing for ripe females (stage IV and V, described by Yano et al. 1988) began 2 wk after feeding was initiated and was conducted daily at 1300 h. Ripe females were randomly inseminated by one of two different techniques detailed below.

Double Spermatophore Technique: (used to produce maternal half-sib families)

1) Capture a female with full ovarian development (stage IV-V), visually identifiable by well-developed ovaries which are thick from the posterior edge of the carapace through the posterior end of the abdomen. The ovarian lobes at the base of the carapace should also be fully developed and olive-green in color.

2) Identify a male with fully developed spermatophores and manually eject both spermatophores by applying gentle pressure to the base of the outer corner of the spermatophore until it slips out of the genital pore. Healthy spermatophores show no signs of melanization, are white in color, slightly swelled and are hard to the touch.

3) Carefully hold the ripe female so that her thelycum is exposed. The fourth and fifth sets of pereopods should be directed posteriorly and held against the ventral surface of her abdomen. Dry the exposed thelycum by blotting it with a paper towel.

4) Place the first spermatophore anterior to the thelycum between the base of the third and fourth pereopods perpendicular to the long axis of the body. Return the fourth set of pereopods to their normal position, securing the first spermatophore in place. Place the second spermatophore posterior to the thelycum between the base of the fourth and fifth pereopods perpendicular to the long axis of the body. Return the fifth set of pereopods to their normal position, securing the second spermatophore in place. Using an index finger, spread the glutinous material surrounding the spermatophore structure to cover the thelycum. Place the female in a spawning tank overnight. The insemination process should be completed in less than 1 min to reduce stress to the female.

Single Spermatophore Technique: (used to produce paternal half-sib families)

The procedure for the single spermatophore technique is identical to that of the

Figure 1. Liberating sperm mass from the spermatophore of a male *L. vannamei* broodstock.

double spermatophore technique through step 3.

4) Place a single spermatophore between the thumb and index finger with firm constant pressure being applied from the bottom (closed end) toward the top (open end) of the spermatophore. This pressure ruptures the sperm sac and liberates a sperm mass that forms a droplet between the thumb and index finger. It also separates the sperm mass from a sheath of glutinous material and the spermatophore. Using angled forceps, remove the sperm mass so that the droplet sits on top of the closed tip of the forceps (Fig. 1).

5) Hold the female securely in the position described in step 3 and carefully place the sperm mass inside the thelycum by inverting the forceps (Fig. 2). The thelycum serves as the seminal receptacle and is enclosed by the coxae of the third and fourth set of pereopods and also partially by the ventral setae of these structures (Dall et al. 1990). After the sperm mass is correctly positioned, return the pereopods to their normal position, which helps to "lock in" the sperm mass,

Figure 2. Placement of sperm mass into the thylecum of a female *L. vannamei* broodstock.

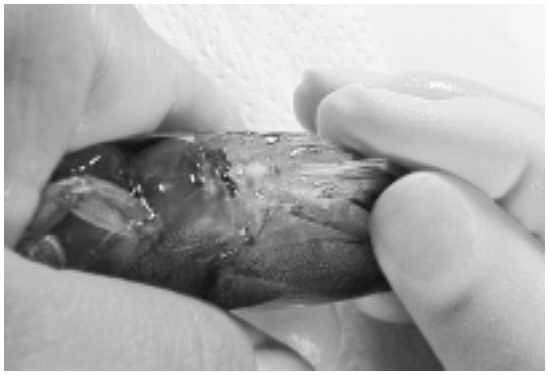

Figure 3. Completed artificial insemination via single spermatophore technique.

and place the female in a spawning tank overnight (Fig. 3). This process should be completed in less than 1 min to reduce stress to the female.

Prior to insemination, all spawning tanks were filled with 300 L of filtered sea water at 29 C and aerated with a 2-cm air stone. A solution of 10 ppm EDTA (disodium salt, Sigma Chemical, St. Louis, MO, USA) at a volume 150 ml was added to each spawning tank. Tanks were checked for eggs the following morning at 0800 h and females were returned to the maturation tanks. In tanks containing successful spawns, aeration was increased to promote gentle mixing of the water column and eggs were allowed to hatch for 2-3 h. Between 1000 and 1100 , air stones were removed and viable nauplii were collected using a light attached to the side of the spawning tank. Positively phototactic nauplii were harvested by siphoning into a 10-L bucket and rinsed with fresh sea water. Nauplii were sampled using a 10-ml Hensen-Stemple pipette (Wildlife Supply Company, Saginaw, MI, USA) and counted volumetrically. A successful spawning event was defined by the production of at least 3,000 viable nauplii by a single female. Spawning success and number of viable nauplii produced per spawn were compared between the two AI techniques with ANOVA using SAS version 6.12.

RESULTS AND DISCUSSION

The single spermatophore technique resulted in a significantly higher ($P < 0.001$) spawning success (84%; n = 120) than the double spermatophore technique (58%; n = 133), and it produced significantly more ($P < 0.001$) viable

nauplii per spawn (24,400 nauplii *vs* 8,500 nauplii). The number of half-sib families produced with the single spermatophore technique also increased. The ratio of full-sib families to number of sires was 1.7 for the single spermatophore technique, whereas the ratio of full-sib families to number of dams was 1.2 for the double spermatophore technique. In addition, the time it took to produce 80 families decreased from 14 d to 9 d. In a recent breeding run using only the single spermatophore technique, the mean number of viable nauplii per spawn was 36,500, spawning success was 87%, and the half-sib ratio was 1.7.

An important distinction between the two AI techniques described above is that, in the single spermatophore technique, the sperm sac is ruptured thereby liberating the sperm mass from the chitinous spermatophore. Also, in this procedure, the sperm is separated from a sheath of glutinous material surrounding the spermatophore that may interfere with fertilization. Using forceps to apply the sperm mass inside the thelycum allows for more effective fertilization of eggs and increases the number of viable nauplii. In addition, using a single spermatophore per female is a more efficient use of male gametes and increases the number of half-sib families because it precludes the need for a specific female to spawn twice in a 2-wk period.

One advantage of producing an increased number of viable nauplii in a shorter time is that potential confounding environmental effects are minimized. Increasing the number of half-sib families is advantageous to a breeding program because it allows for more accurate estimates of genetic parameters, such as heritability and genetic correlations. Heritability describes the percentage of phenotypic variance that is inherited in a predictable manner and is used to determine the potential response to selection. Heritability estimates may be used to estimate progress in a breeding program, whereas phenotypic and genotypic correlations may reveal an indirect response, either positive or negative, to a breeding plan. Use of the single spermatophore AI technique and production of paternal half-sib families has improved the efficiency of the breeding program at OI and should be considered in other shrimp breeding programs.

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